

# **A Semi-Synthetic Method for Synthesis of SUMOylated Hypoxia Inducible Factor-1 $\alpha$ for Future Functional Analysis**

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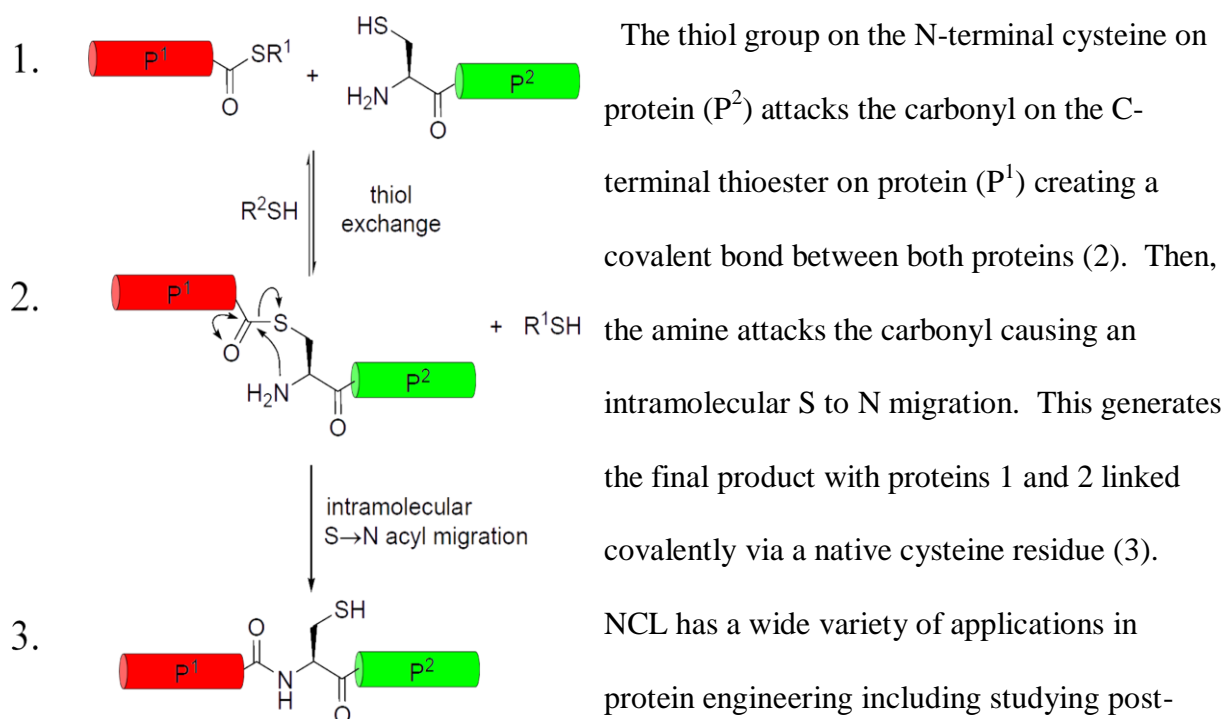
**Abstract:** Native chemical ligation (NCL) is a method used to synthesize a covalent bond between two proteins. The method has been extensively used to react a C-terminal thioester on one protein with an N-terminal cysteine residue on another protein. Our strategy deviates from this usual method by incorporating a cysteine containing analogue of pyrrolysine (PCA) into any site on a recombinant protein. My work uses NCL to conjugate Small Ubiquitin like Modifier (SUMO) to Hypoxia Inducible Factor -1 $\alpha$  (HIF-1 $\alpha$ ) prelabeled with PCA.

HIF-1 $\alpha$  is a transcription factor that is known to generate a protective response during hypoxia, a common symptom of stroke in which there are depleted oxygen levels in tissues. Furthermore, during hypoxia, SUMO is conjugated to HIF-1 $\alpha$  at Lys<sup>391</sup> and Lys<sup>477</sup>. An *in vitro* preparation of SUMOylated HIF-1 $\alpha$  is desired because of two challenges in isolating the protein from *in vivo* preparations; first, HIF-1 $\alpha$  is rapidly degraded in normal respiring tissues and second, deconjugating enzymes cleave SUMO away once the function of the SUMOylated form is mediated in the cell. In order to generate the *in vitro* conjugated product we use the principles of intein chemistry to generate a thioester on SUMO. We incorporate PCA at previously predicted SUMOylation sites and set up a reaction with the SUMO derived thioester. We purified the conjugated products and this conjugated product will be utilized to create better anti-ischemia agents for patients with strokes or metabolic disorders that require high oxygen demands.

## Introduction:

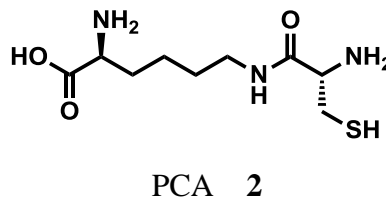
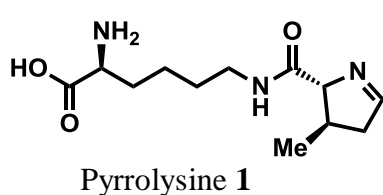
Native chemical ligation (NCL) is one of the most useful techniques for synthesizing a native peptide bond between two unprotected proteins or large peptides [1, 2]. The chemistry involves a C-terminal thioester reaction with a thiol group on cysteine generating a native bond between the two reacting proteins [2]. The overall reaction mechanism of cysteine based NCL is shown in Figure (1).

Figure 1:



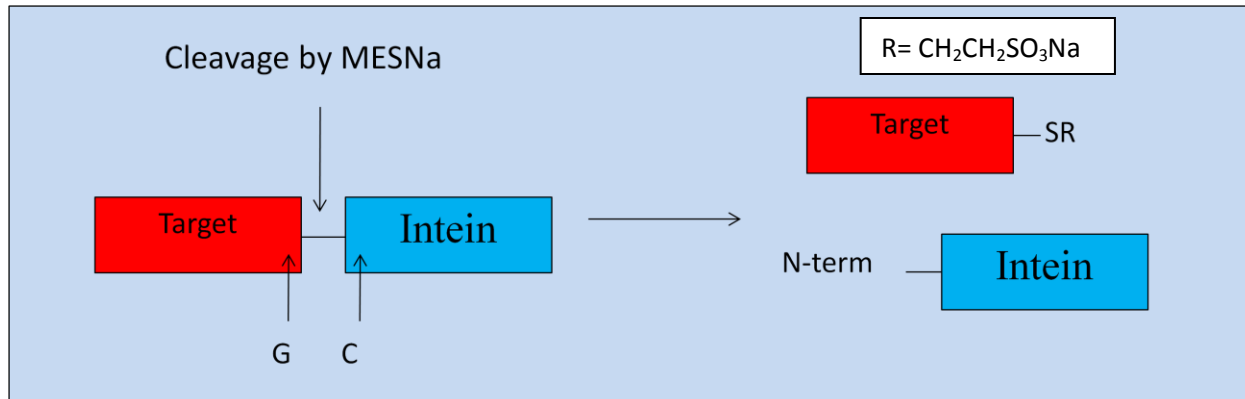
translational modifications, peptide cyclization, and peptide construction. In this present study we introduce a genetically encoded pyrrolysine analogue that places a ligation handle directly into a recombinant protein that can be used for NCL [3]. Pyrrolysine **1** is the 22<sup>nd</sup> amino acid introduced during translation via a UAG codon [4]. Similarly, D-Cysteine containing pyrrolysine analogue (PCA) (*S,S*)-**2** (D-Cys- $\epsilon$ -Lys) has been shown to read through the UAG codon with high efficiency [3]. Through NCL this ligation handle in PCA is allowed to react

with a C-terminal derived thioester generated via intein mediated cleavage by sodium 2-mercaptoethanesulfonate (MESNa) [5].



MESNa cleaves specifically between a C-terminal glycine residue on the target protein and an N-terminal cysteine residue on intein [5]. When intein is fused to and coexpressed with the target protein, a C-terminal thioester can be generated after cleaving with MESNa (Figure 2). In this study we use intein chemistry and PCA mediated NCL to ligate Hypoxia Inducible Factor -1 $\alpha$  (HIF-1 $\alpha$ ) to Small Ubiquitin like Modifier (SUMO) for future functional analysis.

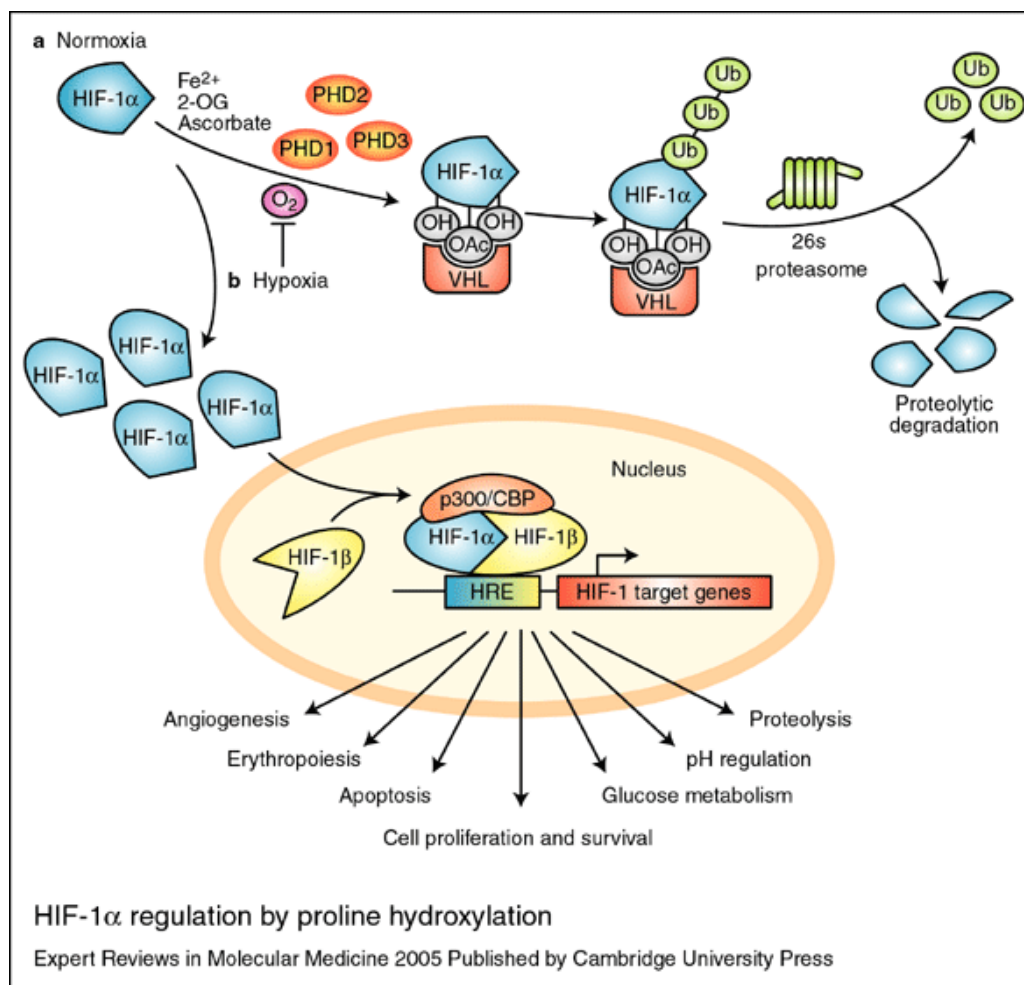
Figure 2:



HIF-1 $\alpha$  is a transcription factor that is known to generate a protective response during hypoxia, a common symptom of stroke in which there are depleted oxygen levels in tissues. In a normoxic state, two conserved proline residues in HIF-1 $\alpha$  are hydroxylated by prolyl hydroxylase and rapidly targeted for proteasome-mediated degradation through the E3 ubiquitin ligase complex which requires the recognition of von Hippel Lindau (VHL) [6-8]. However

during hypoxia, the hydroxylation of HIF-1 $\alpha$  is blocked thereby stabilizing the protein for downstream transcription activity. In a hypoxic response HIF-1 $\alpha$  is translocated to the nucleus where it dimerizes with constitutively expressed HIF-1 $\beta$  [9]. This dimer is then recognized by the coactivators p300/CBD and binds to hypoxia response elements (HREs), promoting the transcription of specific genes and eliciting responses such as glycolysis, erythropoiesis, angiogenesis and vascular remodeling (Figure 3) [9].

Figure 3: Adapted from [10]



Furthermore, during hypoxia HIF-1 $\alpha$  is conjugated to Small Ubiquitin-like Modifier (SUMO) at Lys<sup>391</sup> and Lys<sup>477</sup> [12]. SUMO is a small 12 kDa protein that is conjugated to many proteins

post-translationally via a lysine residue to modify their functions. However, the role of sumoylation in HIF-1 $\alpha$  is vaguely understood because HIF-1 $\alpha$  is rapidly degraded in normal respiring tissues, and Sentrin-specific proteases (SENPs) cleave SUMO away once the function of the SUMOylated form is mediated in the cell [11]. Therefore, an *in vitro* preparation of SUMOylated HIF-1 $\alpha$  is desired. To do this we cloned HIF-1 $\alpha$  into a vector containing pyrrolysine's tRNA (pylT) and tRNA synthetase (pylS). We mutate either Lys<sup>391</sup> or Lys<sup>477</sup> in HIF-1 $\alpha$  to TAG, so PCA can be incorporated into the protein Figure (4). We also cloned SUMO-Intein-CBD into the PRSF vector Figure (5).

Figure 4:

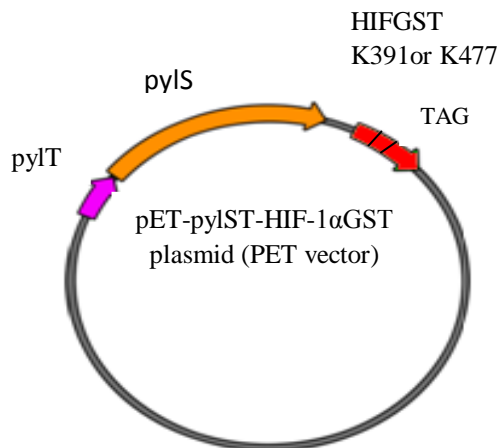
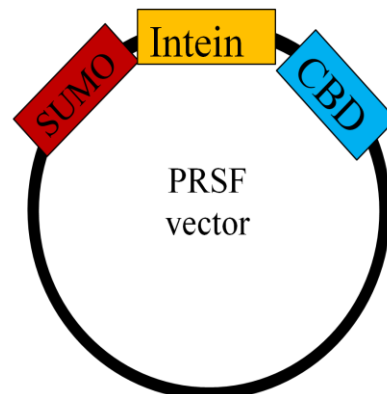


Figure 5:



Once SUMO-Intein-CBD is purified and cleaved with MESNa, a thioester on SUMO is generated and can react via NCL with PCA labeled HIF-1 $\alpha$ GST at the desired SUMOylation sites. This *in vitro* conjugated product can be employed to study functional analyses in order to better understand the role of SUMO on HIF-1 $\alpha$ .

## Methods:

## **Induction and Purification of His-SUMO-Intein-CBD Fusion Protein**

The pRSF vector was purchased from Novagen and His-SUMO-Intein-CBD was cloned into pRSF by Dr. Manoj Nair. The Histag-SUMO-Intein-CBD plasmid was transformed into BL-21 competent cells (*E. coli*) and grown on a kanamycin (kan) resistant LB agar plate at 37 °C for approximately 18 hours. Transformed cells were inoculated into 500 ml 2xYT bearing a final kanamycin concentration of 100 µg/ml and incubated at 37 °C and 250 rpm until the OD<sub>600</sub> reached ~ 0.5. Isopropyl-β-D-1 thiogalactopyranoside (IPTG) (1mM) was added to the media and incubated at 25 °C overnight at 250 rpm. The cells were centrifuged for 5 minutes at 4 °C, 8,000 rpm. The cells were separated in two 100 ml plastic jars and resuspended in 50 ml 1X phosphate buffer (200 mM NaCl, pH 8.0). Benzamidinium- HCl (1 mM) and phenylmethylsulfonylfluoride (PMSF) (1 mM) were added to the solution and the cells were lysed by sonication. This solution was centrifuged for 15 minutes at 4 °C, 10,000 rpm and the supernatant was filtered using 0.45 µm Ministart filter units. The protein was purified by affinity chromatography by binding to 5 ml nickel resin [Clontech] and incubating for 1 hour at 4 °C. The SUMO-Intein-CBD was eluted by using an imidazole gradient (0-1M). Benzamidinium HCl was added to each elution to a final concentration of 1 mM.

## **Cleavage of His-SUMO-Intein-CBD and Purification of His-SUMO-SR**

Sodium 2-mercaptoethanesulfonate (MESNa) (100 mM) was added to the purified protein sample and incubated at room temperature for 48 hours to create a thioester on the C-terminus of His-SUMO. The Intein-CBD was removed by binding to 5 mL chitin beads [BioLabs] and the resulting flow through was dialyzed against ligation buffer (10 mM HEPES, 200 mM NaCl, pH 8.45). After dialysis, His-SUMO-SR was bound to 5 mL nickel resin [Clontech] by incubating

for 1 hour at 4 °C. The protein was eluted using an imidazole gradient (0- 1M). Benzamidine-HCl, MESNa and Tris (2-Carboxyethyl) phosphine Hydrochloride (TCEP) were added to each elution in a final concentration of 1 mM for storage.

### **Induction and Purification of pETpylST-HIF-1 $\alpha$ GST Mutants K391x and K477x**

The pET vector was purchased from Novagen and pylST was cloned into the vector by Dr. Marianne Lee. HIF-1 $\alpha$ GST was cloned into the pET-pylST plasmid by Dr. Manoj Nair. HIF-1 $\alpha$ GST was mutated at K391x or K477x to TAG. The induction and purification of K391x and K477x were performed separately but their procedures were identical. The pET-pylST-HIF-1 $\alpha$ GST (K391x or K477x) plasmid was transformed into BL-21 competent cells (E.coli) and incubated on two ampicillin bearing agar plates at 37 °C for approximately 18 hours. Transformed cells were inoculated into 100ml 2XYT media bearing 100  $\mu$ g/ml of ampicillin and incubated at 37 °C and 250 rpm until the OD<sub>600</sub> reached 0.6. PCA (synthesized by Dr. Tomek Fekner) was added to the media at a final concentration of 5mM and the pH of media was restored to ~7.5 using 1M Tris base. IPTG (1 mM) was added to all four flasks and then incubated at 25 °C with shaking at 250 rpm overnight. The cells were centrifuged in a 50 ml falcon tube (1 falcon tube/ flask) at 8,000 x g at 4 °C for 10 minutes. The resulting pellets were resuspended in 25 mL HEPES buffer (10 mM HEPES, 0.5 M NaCl, 1 mM TCEP, 1 mM benzamidine - HCl pH 8.0) and lysed by sonication. The remaining cells were centrifuged at 10,000 rpm for 10 minutes and the supernatant was bound to GST resin by incubating for 1 hour at 4 °C. The column was washed by incubating with 4 mL GST wash buffer (30 mM glutathione, 200 mM NaCl, 10 mM HEPES, 1 mM TCEP, 1 mM benzamidine- HCl) for 5 minutes at 4 °C. The protein was eluted by incubating with 3 mL GST elution buffer (100 mM glutathione, 200 mM NaCl, 10 mM HEPES, 1 mM TCEP, 1 mM benzamidine- HCl) for 15



minutes at 4 °C. This step was repeated until no blue color was shown on a Bradford analysis. Benzamidine HCl and TCEP were added to each elution at a final concentration of 1 mM for storage.

### **Ligation of His-SUMO-SR to HIFGST Mutants K391x and K477x and purification of SUMOylated Products:**

Mutants K391x and K477x share the same procedure but were prepared separately. The HIF-1 $\alpha$ GST 100 mM elutions were concentrated using a 30K cutoff centrifugal filter unit [Millipore] and dialyzed against ligation buffer (10 mM HEPES, 200 mM NaCl, pH 8.45). His-SUMO-SR was concentrated using a 3K cutoff [Millipore] and dialyzed against ligation buffer. The concentrations were measured using a Bradford analysis and SUMO-SR was added to the 100 mM concentrated sample based on a 2:1 mass ratio SUMO: HIF. Benzamidine –HCl was added in a final concentration of 2 mM. The tube was incubated at room temperature with moderate mixing for 36 hours. GST resin (200  $\mu$ l) was washed with 10 -X CV of water and then 10-X CV of ligation buffer. The ligation sample was added to the resin and incubated at 4 °C for 2 hours. The resin- protein mixture was placed into a spin column and centrifuged for 1 minute at 1000 x g. After washing the resin with ligation buffer, GST elution buffer (100 mM glutathione, 200  $\mu$ l) was added to the spin column and incubated at 4 °C for 30 minutes. The eluate was collected by centrifugation at 1000 x g for 1 minute. This process of incubating with GST elution buffer was repeated until no blue color was seen using a Bradford analysis. Nickel resin (200  $\mu$ l) was washed with 10 -X CV of water and then 10-X CV of ligation buffer. The elutions from the GST resin were pooled and incubated with the nickel resin at 4 °C for 2 hours. The resin protein mixture was placed into a spin column and centrifuged for 1 minute at 1000 x g. The pure ligated product was eluted by incubating for 15 minutes with increasing concentrations of

imidazole (0- 1M) in a stepwise fashion. The elutions were collected by centrifugation for 1 minute at 1000 x g.

## **Results:**

### **Generation of Thioester on SUMO**

In order to SUMOylate HIF-1 $\alpha$  *in vitro* we must first generate a thioester on SUMO. Our plasmid contains the SUMO-Intein fusion protein with a C-terminal Histag on SUMO and an N-terminal chitin-binding domain (CBD) on Intein. First, we purify the Histag-SUMO-Intein-CBD via affinity chromatography by binding to nickel resin. The bulk of the protein eluted at 200 mM and 500 mM imidazole as shown on Figure (6). The purified SUMO-Intein was cleaved with MESNa and most of the Intein-CBD and full length protein were removed by binding to chitin beads Figure (7). The presence of Intein-CBD and full length protein indicate that the binding to chitin was insufficient however, the washes are much cleaner. We pooled washes 1 and 2 from Figure (7) and we purified via affinity chromatography by binding to nickel resin. Once again, the resulting protein was eluted at 200 mM and 500 mM imidazole Figure (8). It may also be noted that 1 mM MESNa and 1 mM TCEP were added for storage to prevent SUMO-SR oxidation.

Figure 6:

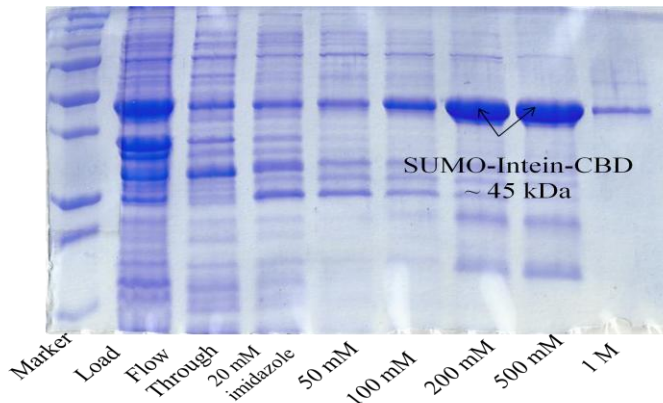


Figure (6) shows the SDS-PAGE of the SUMO-Intein purification. The bulk of our protein eluted at 200 mM and 500 mM imidazole.

Figure 7:

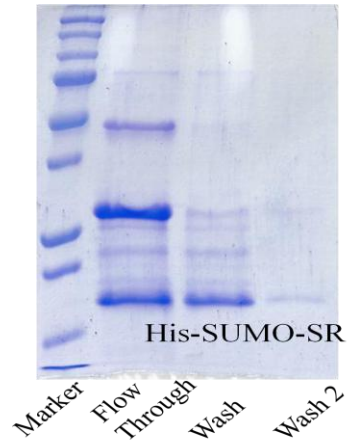


Figure (7) shows the SDS-PAGE after cleavage with MESNa. Most of the Intein-CBD and full length protein was removed by binding to chitin beads.

Figure 8:

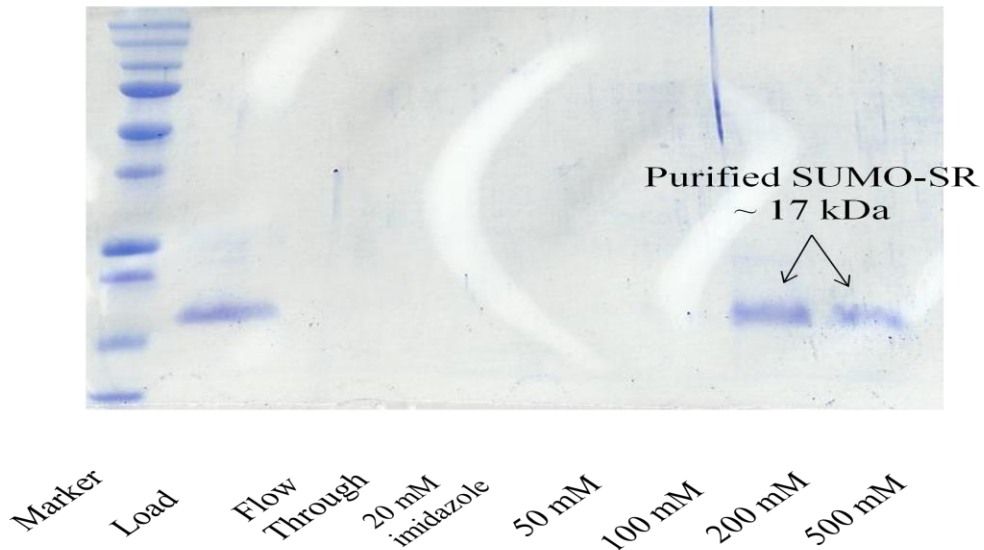


Figure (8) shows the SDS-PAGE of the SUMO-SR. Our protein eluted at 200 mM and 500 mM imidazole as one solid band.

### Purification of HIFGST K391x and K477x

We purified both mutants separately, but the procedure was identical. The mutants were purified via affinity chromatography and eluted using 30 mM and 100 mM glutathione. Some of our protein eluted at 30 mM glutathione, but it was contaminated with other proteins. Then, both mutants were eluted using 100 mM glutathione for cleaner results. The SDS-PAGE of mutants K391x and K477x are shown in figures (9) and (10) respectively and are running at ~ 60 kDa in size. To confirm the identity of the mutants we performed a Western Blot using an anti-GST antibody Figure (11). The result shows purified HIF-1 $\alpha$ GST K391x and K477x around the approximate size shown on the SDS-PAGE.

Figure 9:

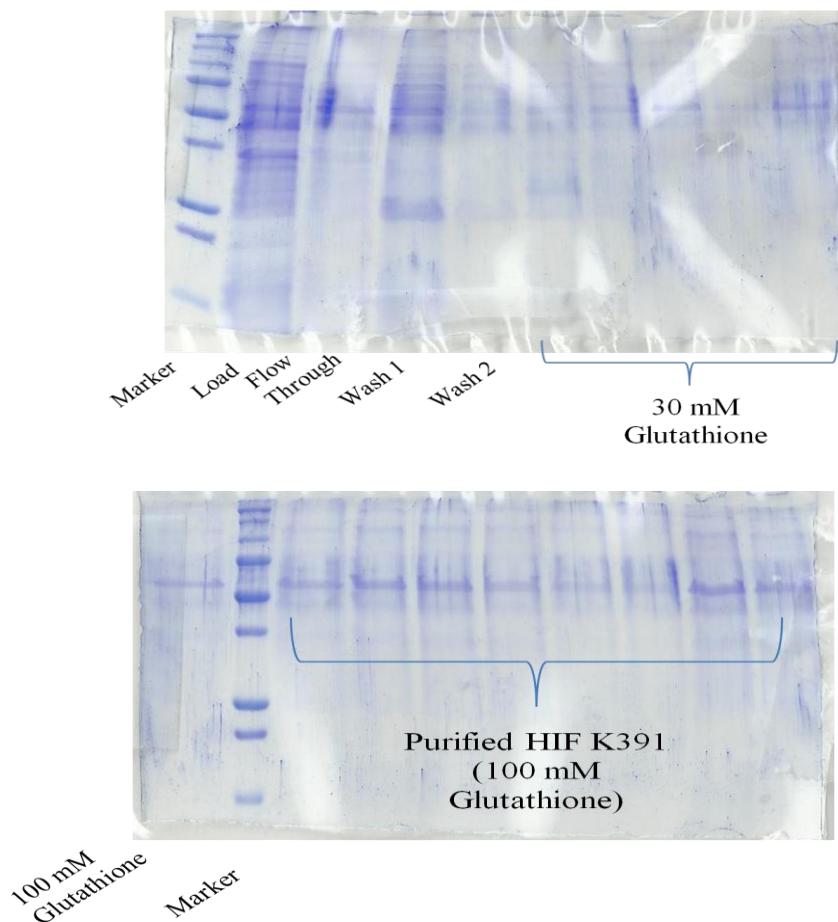


Figure (9) is an SDS-PAGE of HIF-1 $\alpha$ GST mutant K391X. Some of our protein eluted at 30 mM glutathione but was dirty. After most of the impurities were washed away with 30 mM glutathione our pure protein was eluted with 100 mM glutathione. HIF-1 $\alpha$ GST was running at ~ 60 kDa.

Figure 10:

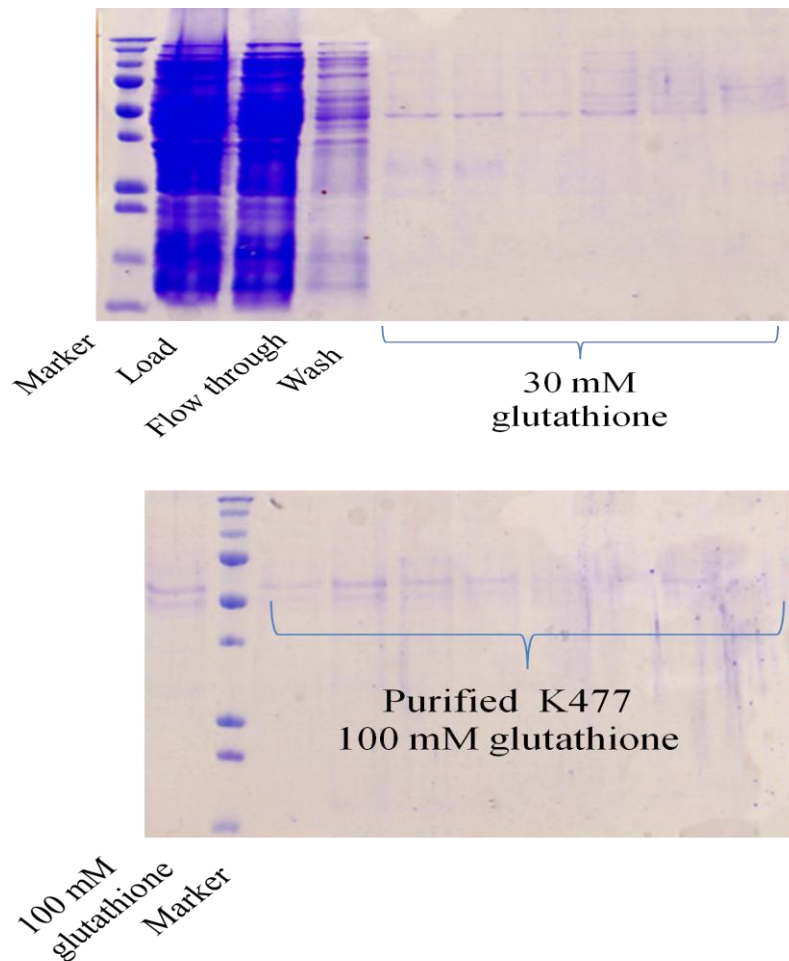


Figure (10) is an SDS-PAGE of HIF-1αGST mutant K477X. Some of our protein eluted at 30 mM glutathione but was dirty. After most of the impurities were washed away with 30 mM glutathione our pure protein was eluted with 100 mM glutathione. HIF-1αGST K477x was running at ~ 60 kDa.

Figure 11:

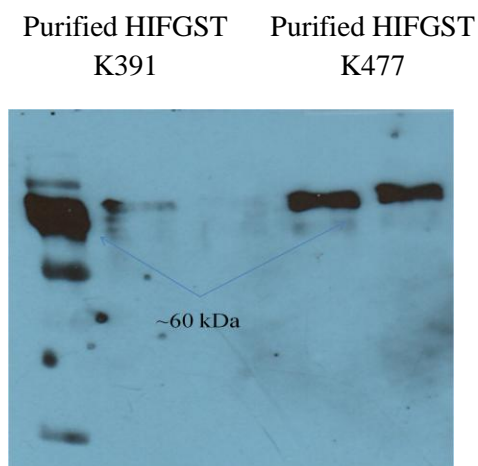


Figure (11) shows a Western blot of purified K391 and K477. The antibody used here was an anti-GST antibody. There appears to be mild degradation of mutant K391.

## **Purification of Ligated Products K391 and K477**

The K391x and K477x mutants were purified separately but by the same procedure. Mutants K391x and K477x were first purified via GST affinity chromatography. Figure (12) shows the SDS-PAGE of K391x after GST purification and figure (14) shows the SDS-PAGE of K477x after GST purification. All of the SUMO-SR that did not react with HIF-1 $\alpha$  did not bind and is seen in the flow through in both of these figures. Next, the elutions were pooled and purified via nickel resin. Figure (13) shows the SDS-PAGE of K391x after Histag purification and figure (15) shows the SDS-PAGE of K477x after Histag purification. The ligated product runs at ~ 75 kDa. To confirm that this 75 kDa band corresponds to the ligated product we performed a Western blot using anti-SUMO and anti-HIF antibodies. We did not have enough ligated K477x protein left after purification to do a clean western blot, but we did have enough to test one for ligated K391x. Figure (16) is an anti-SUMO Western blot of pure ligated K391x. Figure (17) is an anti-HIF Western blot of ligated K391x before purification. I am in the process of creating an anti-HIF Western of pure ligated K391x.

## Purification of K391 Ligated product

Figure 12:

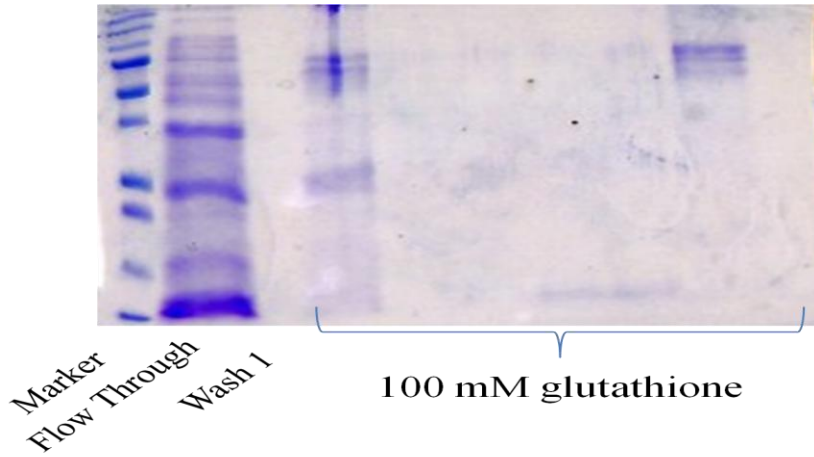


Figure (12) shows the SDS-PAGE of ligation purification of K391x after GST purification.

Figure 13:

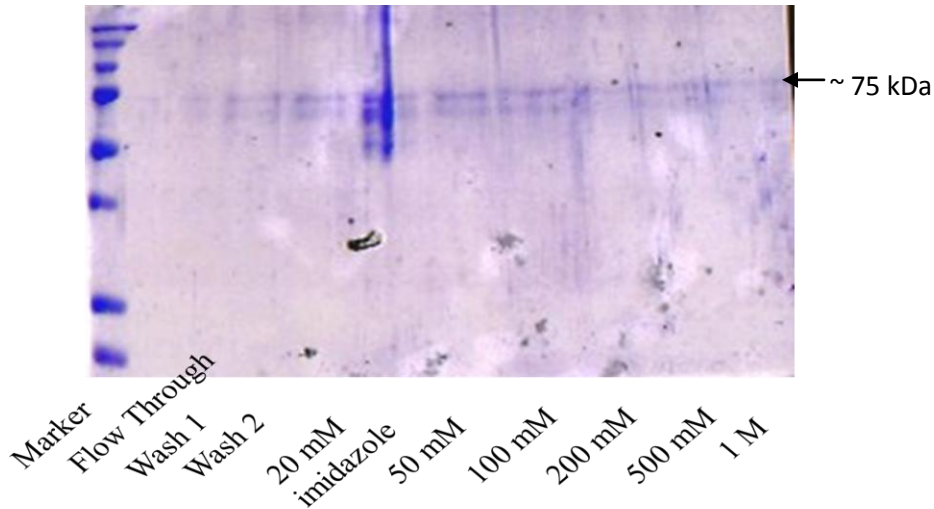


Figure (13) is an SDS-PAGE of ligated K391x after nickel purification. The protein eluted at all concentrations of imidazole. The ligated K391x product runs at ~ 75 kDa.

## Purification of K477 Ligated Product

Figure 14:

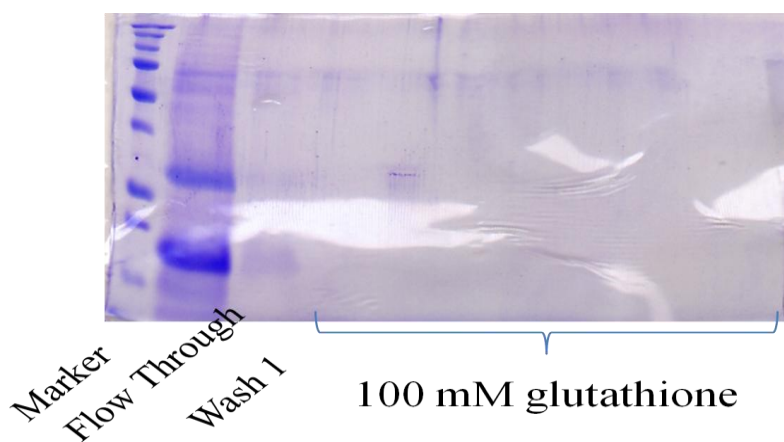


Figure (14) shows the SDS-PAGE of ligation purification of K477x after GST purification.

Figure 15:

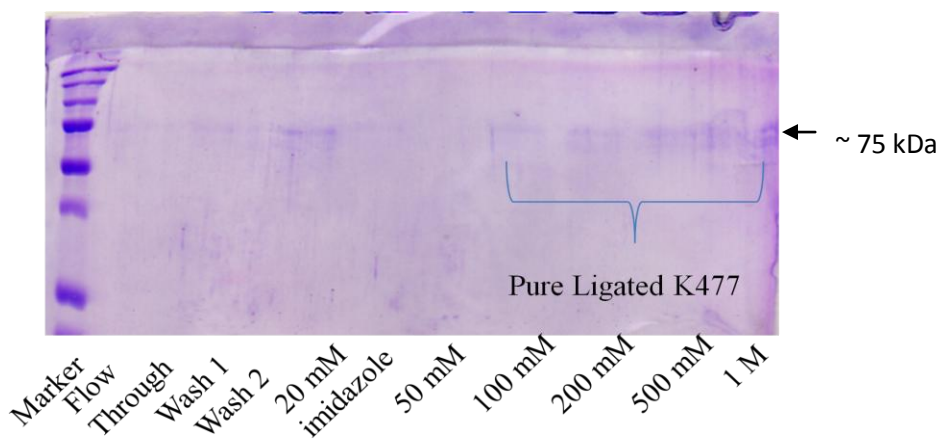
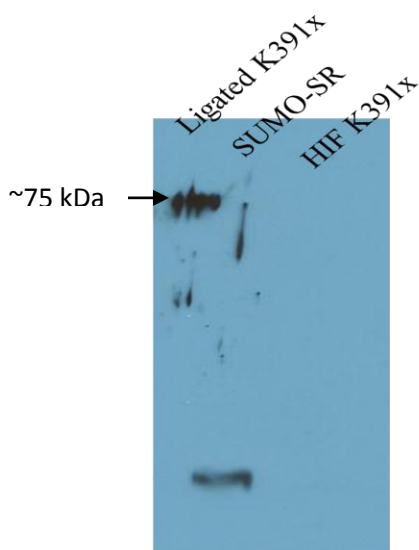


Figure (15) is an SDS-PAGE of ligated K477x after nickel purification. The protein eluted at all concentrations of imidazole. The ligated K477x product runs at ~ 75 kDa.



**Figure 16:**



**Figure 17:**

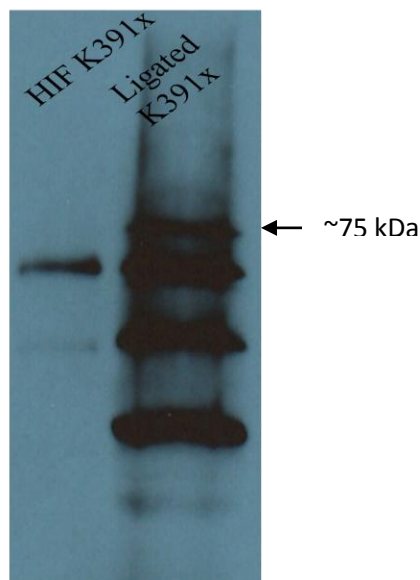


Figure (16) is an anti-SUMO Western blot of pure ligated K391x and Figure (17) is an anti-HIF Western blot of ligated K391x before purification.

### **Discussion:**

Although NCL is widely used in connecting two proteins via a cysteine residue, the uses of NCL have been limited to a thioester reaction with an N-terminal cysteine [13-15]. This hinders the ability to utilize NCL site specifically because the reaction is restricted to a C to N-terminus ligation. Herein we deviate from the usual method of NCL by utilizing our pyrrolysine analog PCA. This allows us to site specifically incorporate PCA into a protein which can then react with a C-terminal thioester by NCL. This greatly increases the uses of NCL because we now have multiple potential positions that can undergo NCL, and we are not limited to an N-

terminal cysteine. Therefore, any post-translational modification in which another protein or small molecule is attached via a lysine residue can be studied using PCA mediated NCL. These include sumoylation, ubiquitination, biotinylation, succinylation and many more. To investigate uses of PCA mediated NCL, we synthesized SUMOylated HIF-1 $\alpha$ .

The first step in elucidating the function of SUMO on HIF-1 $\alpha$  is an *in vitro* synthesis of SUMOylated HIF-1 $\alpha$ . As I mentioned earlier, the role of sumoylation in HIF-1 $\alpha$  is vaguely understood because HIF-1 $\alpha$  is rapidly degraded in normal respiring tissues, and SENPs cleave SUMO away once the function of the SUMOylated form is mediated in the cell [11]. This hinders the ability to study the effect of SUMO on HIF-1 $\alpha$  *in vivo*. However, with PCA mediated NCL we have successfully created the *in vitro* conjugated products at the two sumoylation sites. The increase from a 60 kDa band in the HIF-1 $\alpha$ GST purifications to a 75 kDa band in the SUMOylated HIF-1 $\alpha$ GST purifications signifies that SUMO, which is running at 17 kDa, is ligated to HIFGST in both mutants. The Western blot of K391x also confirms the presence of the ligated product because it reacted with the anti-SUMO and anti-HIF antibodies.

Now that we have a stable conjugated product we can begin to identify the role sumoylation plays in HIF-1 $\alpha$ . There are multiple potential functions SUMO could have during hypoxia. SUMO could block the function of prolyl hydroxylase, leading to HIF-1 $\alpha$  stabilization and downstream transcription activity. Moreover, SUMO could aid in the translocation of HIF-1 $\alpha$  to the nucleus, or assist in DNA binding. SUMO could have one or more of these functions and to fully understand SUMO's role during hypoxia all of these potential functions must be tested. Furthermore, it is possible that both K391x and K477x sites need to be SUMOylated in order to carry out its function(s) during hypoxia. We have a plasmid with HIF-1 $\alpha$ GST mutated at sites K391x and K477x, and we have tested the expression (data not shown). However, we

need to purify the double mutant and set up a ligation reaction with SUMO. With the availability of the various forms of HIF1 $\alpha$  (conjugated to PCA at one or multiple sites), we plan to probe the role of this post translation modification in the mechanism of action of HIF1 $\alpha$ .

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